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OPTIMIZATION OF HIGH QUALITY RNA ISOLATION METHOD FROM *STEVIA REBAUDIANA* BERTONI LEAF FOR Q-PCR ANALYSIS

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ABSTRACT

A suitable method has been optimized to isolate high quality RNA from polyphenols and glycosides rich leaves of *Stevia rebaudiana* for Q-PCR analysis. RNA isolation protocol was standardized through some modifications of standard guanidinium isothiocyanate – phenol based method, CTAB-LiCl based method and tris - saturated phenol based method. Quality and yield of isolated RNA were assessed through spectrophotometric technique, band profile in 1.5% (w/v) non-denaturing agarose gel electrophoresis and amplification efficiency during Q-PCR analysis. Modification of standard guanidinium isothiocyanate – phenol based RNA isolation method through addition of 2% (w/v) polyvinyl pyrrolidone and 2% (w/v) β-mercaptoethanol during extraction followed by chloroform: isoamyl alcohol (24:1) induced phase separation and precipitation in 8M lithium chloride was found to be best with respect to quality, quantity and downstream application of isolated RNA. The proposed protocol is indeed promising to isolate high quality RNA from secondary metabolites rich *S. rebaudiana* leaves.

Keywords : RNA isolation, Q-PCR, *Stevia rebaudiana*

साराँश

पोलीफेनोल एवं ग्लाइकोसाइड प्रचुरयुक्त *स्टीविया रिबाउडियाना* की पत्तियों से उत्तम श्रेणी के आर०एन०ए० प्राप्त करने की उपयोगी विधि का वर्णन प्रस्तुत किया गया है ।

Introduction

Exploration of gene expression and regulation through Q-PCR analysis requires a pure and un-degraded RNA. Various protocols have been developed or some time extensively modified by several researchers for the isolation of high-quality RNA from plant tissues. There are several factors which found to affect the RNA quality during extraction like RNAse degradation, presence of high levels of phenolic compounds and polysaccharides in the plant tissue (Kansal et al., 2008). Moreover, the formation of phenol-quinone (oxidized product of phenolic compounds) complexes in the extracts that bind to RNA as well as the co-precipitation of polysaccharides with RNA in low-ionic strength buffers hinder pure quality RNA isolation and it's further applications (Birtic and Kranner 2006; Manickavelu et al., 2007). Therefore, presence of polysaccharides, phenols and other secondary metabolites like polyphenols, terpenoids significantly interfere with the extraction and purification of nucleic acid that ultimately hamper the quality. Such poor quality RNA eventually leads to erroneous quantification and interferes with reverse transcription as well as PCR amplification (Koonjul et al., 1999).

The quality and quantity of extracted RNA is absolutely inconsistent and long term storage renders instability for downstream application (Bustin et al., 2005; Pfaffl, 2005a). Long RNA fragments often found to be very susceptible to degradation due to enzymatic cleavage during extraction and storage. Therefore, RNA preparation should be meticulously evaluated for maintaining high quality and quantity. Several RNA isolation methods from plant tissue with high level of secondary metabolites were reported. However, most of the methods found to be tissue specific and suitable for particular groups of plant and only a few have been reported for a wide range of plant species with certain limitations. Extraction of RNA by guanidinium isothiocyanate – phenol - chloroform based method is found to be the most common technique for a wide range of species. One of the most important and available guanidinium isothiocyanate – phenol based RNA extraction reagent is TRIZOL (Invitrozen, USA), which was applied for rapid RNA isolation in species such as *Arabidopsis* (Box et al., 2011) and rapeseed (Pant et al., 2009). However, TRIZOL reagents have proved unsuitable for extracting RNA from plant tissues rich in polysaccharides and polyphenolics (Tattersall et al., 2005). Isolation of RNA through guanidinium– phenol–chloroform based extraction was also found to be unsuitable for leguminous plant materials rich in polyphenols (Baker et al., 1990). Extraction of RNA from plant using CTAB (cetyltrimethylammonium bromide) buffer followed by LiCl (lithium chloride) precipitation was reported by Alemzadeh et al., (2005). Moreover, addition of PVP (polyvinyl pyrrolidone), β – mercaptoethanol in the extraction buffer was found to be effective in elimination of contaminants during RNA extraction (Valenzuela-Avendaño et al., 2005).

The present research work is focused on optimization of pure quality RNA extraction from the leaf of *Stevia rebaudiana* and its downstream application in Q-PCR for 26S rRNA gene expression study. *S. rebaudiana* is a well known medicinal plant belongs to the family Asteraceae. *Stevia* is rich in secondary metabolites like diterpenoid glycosides (Starrat et al., 2002), and flavonols such as quercetin, myricetin, isorhamnetin, and kaempferol as well as the corresponding flavones, apigenin (Ghanta et al., 2007). Hence, a fundamental requisite to study gene expression in *Stevia* is isolation of contaminants (secondary metabolites) free pure quality RNA. Standard guanidinium isothiocyanate – phenol – chloroform based method of RNA isolation from *Stevia* was found to be unsatisfactory in terms of quality and quantity of RNA. It was reported that CTAB-LiCl based method of RNA isolation form *Stevia* was good in terms of integrity and purity of isolated RNA (Chao et al., 2009). However, this method is so lengthy that RNA extracted with this method may be degraded some time during the extraction process. Therefore, an efficient method for isolation of pure quality RNA from polyphenols and diterpene glycoside rich *Stevia* leaves was optimized through careful manipulation of above mentioned RNA isolation methods, which subsequently facilitated the gene expression studies.

Experimental procedures

Plant material

Frozen leaves from the *in vitro* grown plantlets of *S. rebaudiana*.

Sterilization of glass and plastic wares

All the autoclavable glass and plastic wares were immersed in 0.1% (v/v) diethyl pyrocarbonate (DEPC) treated water at 37°C overnight and then auto-claved at 15 lb pressure for 30 minutes.

Total RNA extraction

Total RNA extraction protocol was optimized in this work through careful manipulations of standard guanidinium isothiocyanate – phenol

based method; CTAB-LiCl based RNA extraction procedure and tris - saturated phenol method.

Method 1 (Tris-saturated phenol based RNA extraction)

Total RNA was extracted following the method of Kumar et al., (2011) with some modifications. Frozen 100 mg of leaf tissue ground into fine powder using homogenizer with serrated pestle and extracted with 1 ml of tris-saturated phenol based RNA extraction buffer contained phenol saturated with tris (hydroxymethyl) aminomethane buffer (pH 6.7), 0.1% (w/v) sodium dodecyl sulphate , 0.32 M (w/v) sodium acetate and 0.01 M ethylenediaminetetra acetic acid (EDTA, pH 8.0). The extraction was allowed to stand for 5 minutes at room temperature (15-25^{o}C). After that, 200 µl of chloroform : isoamyl alcohol (24:1) was added to the homogenized tissue and shaken vigorously for 20 seconds followed by stand for 10 minutes at room temperature. Then the mixture was centrifuged at 13,000 rpm for 15 minutes at 4^{o}C. Following centrifugation aqueous phase containing RNA was transferred to fresh 1.5 ml eppendorf tube followed by addition of 500 µl of isopropyl alcohol. The mixture was allowed to stand for 5-10 minutes at room temperature (15-25^{o}C) followed by centrifugation at 13,000 rpm for 15 minutes at 4^{o}C. Supernatant was discarded and RNA pellet was washed by addition of 1ml of 75% ethanol. Then the mixture was again centrifuged at 7,500 rpm for 5 minutes at 4^{o}C. RNA pellet was then briefly air dried for 5-10 minutes and dissolved in 50 µl of RNase free water and stored at –80^{o}C in aliquots for further analysis.

Method 2 (CTAB-LiCl based RNA extraction)

RNA extraction was performed using CTAB-LiCl based extraction method of Song et al., (2011) along with some modifications. Frozen 100 mg of leaf tissue ground into fine powder using homogenizer with serrated pestle and extracted with 1 ml of CTAB extraction buffer [3% (w/v) CTAB, 100 mM Tris-HCl (pH 8.0), 1.4 M NaCl, 20 mm EDTA (pH 8.0), 2% (w/v) polyvinyl pyrrolidone (PVP), 2% (w/v) β- mercaptoethanol and 80 µg/ml proteinase K mixed well and incubated at 65°C for 30 minutes with occasional shaking. The supernatant was collected by centrifuging at 10,000 rpm for 10 minutes at room temperature and an equal volume of chloroform-isoamyl alcohol (24:1, v/v) was added, mixed well and centrifuged at 12,000 rpm for 15 minutes at 4°C. The supernatant was collected and 1/3 volume of 8 M LiCl was added, mixed well and incubated at 4°C for overnight. The precipitate was collected by centrifugation at 12,000 rpm for 15 minutes at 4°C and suspended with 200 µl of 10 mM Tris-HCl (pH 7.5) containing 1/10 volume of 3 M potassium acetate (pH 5.5) and kept on ice for 20 minutes, then separated by centrifuging at 12,000 rpm for 15 minutes at 4°C. After centrifugation the RNA pellet was collected, washed twice with 75% (v/v) ethanol and then the mixture was again centrifuged at 7,500 rpm for 5 minutes at 4^{o}C. RNA pellet was then briefly air dried for 5-10 minutes and dissolved in 50 µl of RNase free water and stored at –80^{o}C in aliquots for further analysis.

Method 3 (Guanidinium isothiocyanate based RNA extraction)

RNA isolation was performed using guanidinium isothiocyanate based protocol proposed by Wang et al., (2010) along with some modifications. The extraction was carried out using the guanidinium isothiocyanate – phenol based reagent (RNA-Xpress™, Himedia). Details of the extraction procedure are represented below:

Method 3a

Frozen 100 mg of leaf tissue ground into fine powder using homogenizer with serrated

pestle and extracted with 1ml of RNA-Xpress reagent. Then the extract was taken in 1.5 ml eppendorf tube and incubated for 5 minutes at room temperature (15-25°C) to permit the complete dissociation of nucleoprotein complexes. After that, 200 µl of chloroform was added to the homogenized tissue and shaken vigorously for 20 seconds followed by stand for 10 minutes at room temperature. Then the mixture was centrifuged at 13,000 rpm for 15 minutes at 4°C. Following centrifugation, mixture separated into lower deep red organic phase containing protein, an interphase containing DNA and colorless upper aqueous phase containing RNA. The aqueous phase containing RNA was transferred to fresh 1.5 ml eppendorf tube followed by addition of 500 µl of isopropyl alcohol. After that, the mixture was allowed to stand for 5-10 minutes at room temperature (15-25°C) followed by centrifugation at 13,000 rpm for 15 minutes at 4°C. Supernatant was discarded and RNA pellet was washed by addition of 1ml of 75% ethanol. Then the mixture was again centrifuged at 7,500 rpm for 5 minutes at 4°C. RNA pellet was then briefly air dried for 5-10 minutes and dissolved in 50 µl of RNase free water and stored at –80°C in aliquots for further analysis.

Method 3b

Frozen 100 mg of leaf tissue ground into fine powder using homogenizer with serrated pestle and extracted with 1ml of RNA-Xpress reagent containing 2% (w/v) PVP. Then the extract was taken in eppendorf tube and incubated for 5 minutes at room temperature (15-25°C) to permit the complete dissociation of nucleoprotein complexes. After that, 200 µl of chloroform : isoamyl alcohol (24:1) was added to the homogenized tissue and shaken vigorously for 20 seconds followed by stand for 10 minutes at room temperature. Then the mixture was centrifuged at 13,000 rpm for 15 minutes at 4°C. Following centrifugation, mixture separated into lower deep red organic phase containing protein, an interphase containing DNA and colorless upper aqueous phase containing RNA. The aqueous phase containing RNA was transferred to fresh 1.5 ml eppendorf tube followed by addition of 500 µl of isopropyl alcohol. After that, the mixture was allowed to stand for 5-10 minutes at room temperature (15-25°C) followed by centrifugation at 13,000 rpm for 15 minutes at 4°C. Supernatant was discarded and RNA pellet was washed by addition of 1ml of 75% ethanol. Then the mixture was again centrifuged at 7,500 rpm for 5 minutes at 4°C. RNA pellet was then briefly air dried for 5-10 minutes and dissolved in 50 µl of RNase free water and stored at –80°C in aliquots for further analysis.

Method 3c

RNA was extracted with 1ml of RNA-Xpress reagent containing 2% (w/v) PVP and 2% (w/v) β-mercaptoethanol. This method followed the same steps as described in method 3b up to the chloroform: isoamyl alcohol (24:1) induced phase separation. However, here RNA was precipitated using 1/3 volume of 8M lithium chloride (LiCl) instead of isopropyl alcohol and incubated on ice for 1 hour. Then the precipitate was collected by centrifugation at 13,000 rpm for 15 minutes at 4°C. Precipitated RNA pellet was washed by addition of 1ml of 75% ethanol. Then the mixture was again centrifuged at 7,500 rpm for 5 minutes at 4°C. RNA pellet was then briefly air dried for 5-10 minutes and dissolved in 50 µl of RNase free water and stored at –80°C in aliquots for further analysis.

RNA quality and quantity check

The quantity and quality of the total extracted RNA was assessed by determining the absorbance at 260 nm and 280 nm using a UV-Visible spectrophotometer having quartz cuvette of 1 cm pathlength (Systronics,

India). Integrity of the RNA was evaluated by 1.5% (w/v) non-denaturing agarose gel electrophoresis as reported by Sambrook et al., (2000), followed by visualization of ribosomal RNA bands through trans-UV light in gel documentation system (BioRad). Quantity of the RNA was calculated by using the following formula:

Concentration of RNA (μg/ml) = 40 μg/ml × A_{260} × dilution factor.

(For RNA solutions, the absorbance at 260 nm equals to1.0 in 1-cm path length equivalent to 40 μg/ml of RNA)

cDNA synthesis and Q-PCR assay

Synthesis of cDNA was performed using Verso-cDNA kit (Thermo-Fisher) following manufacturer's instructions. The Q-PCR was carried out using a pair of gene specific primers that were designed based on the conserved sequence of the 26S rRNA (Acc. No. AB457301.1) gene from *S. rebaudiana*. The sequences of the primers were: 5'-CACAATGATAGGAAGAGCCGAC-3' (F) and 5'- CAAGGGAACGGGCTTGGCAGAATC- 3' (R) and validity checking was performed using QuantPrime Q-PCR primer designing tool (http:// www.quantprime.de). Polymerase chain reactions were performed in 96-well microtiter plates using SYBR Green (Himedia, India) to detect double stranded DNA synthesis. Amplifications were carried out in a Q-PCR thermocycler (Roche, Light Cycler 48011) using the following cycling parameters: 1 cycle at 95°C for 10 min (pre-incubation); 40 cycles at 95°C for 10 seconds, 55°C for 30 seconds and 72°C for 30 seconds (amplification); 1 cycle at 95°C for 10 seconds, 65°C for 60 seconds (melting) and 1 cycle at 37°C for 10 seconds (cooling). The amplification of the PCR products was analyzed by electrophoresis using a 1% (w/v) agarose gel stained with ethidium bromide and visualized through trans-UV light in gel documentation system (BioRad).

Determination of Q-PCR amplification efficiency

A dilution series (stock; 1:10; 1:100; 1:1000; 1:10000) of respective cDNA samples have been made during Q-PCR analysis. Next, respective diluted cDNA templates were amplified and amplification data were subsequently used to generate a standard curve. The dilution series (X axis) were plotted against C_T values (Y axis) to prepare a linear regression for determination of the correlation coefficient value (R^2), slope and Y-intercept. Amplification efficiency (E) was calculated from the following formula:
$E= 10^{(-1/slope)} - 1$ (Pfaffl, 2001).

Results and Discussion

Appropriate isolation determines the integrity, yield and purity of the RNA, which are crucial for any downstream application like gene expression studies. Therefore, it is desirable to have an efficient RNA isolation method to attain these objectives.

Integrity, quality and quantity of the isolated RNA

The integrity of RNA extracted by different RNA extraction methods was examined by electrophoretic separation on a 1.5% agarose gel (Fig. 1). From the gel image it was noticed that RNA extracted through method 3c (lane 5, fig. 1) and method 3b (lane 4, fig. 1) produced discrete 28S rRNA, 18S rRNA bands. Moreover, integrity of 28S rRNA and18S rRNA bands was more intense in lane 5 as compared with lane 4 (Fig. 1). RNA extracted through methods 1 and 2 developed bands of 28S rRNA and 18S rRNA with less integrity (lane 1 and 3, fig. 1) when compared to that of method 3c. However, invisible bands of respective rRNA in lane 2 indicated the presence of contaminated or degraded RNA that was extracted through method 3a (Fig. 1). Spectrophotometric analysis revealed that the RNA extracted using

method 3c was found to be pure and high quality (Table 1). It was noticed that both the $A_{260/280}$ (2.01 ± 0.15) and $A_{260/230}$ (2.15 ± 0.35) ratios of the RNA extracted using method 3c were above 2 that determines the presence of pure and high quality RNA. Moreover, yield of the total extracted RNA (75.66 ± 2.01 μg/g fresh weight of tissue) was also found to be highest in method 3c in comparison to other RNA extraction methods (Table 1). Furthermore, the $A_{260/280}$ ratio of the RNA extracted through method 1 (1.89 ± 0.51), method 2 (1.81 ± 0.07) and method 3b (1.91 ± 0.18) indicated the presence of protein contaminant in the RNA sample (Table 1). Conversely, the $A_{260/230}$ ratio of the RNA extracted using method 1 (2.05 ± 0.05) and method 3b (2.11 ± 0.50) found to be almost similar to that of method 3c (Table 1). Total RNA yield did not varied significantly in between method1, 2 and 3b (Table 1). In addition, RNA extracted through method 3a was found to be of poor quality, as it was noticed both $A_{260/280}$ (1.72 ± 0.04) and $A_{260/230}$ (1.87± 0.14) ratios were less than two, which confirmed the presence of both protein and organic contaminants in the RNA (Table 1). Total RNA yield (53.34 ± 1.5 μg/g fresh weight of tissue) was found to be lowest in method 3a as compared with the others method (Table 1). Integrity, stability, yield and purity of isolated RNA for gene amplification and Q-PCR analysis determines the suitability of any RNA isolation protocol. In this work RNA extracted through modified of the standard guanidinium isothiocyanate based protocol through addition of 2% (w/v) PVP and 2% (w/v) β-mercaptoethanol in guanidinium isothiocyanate – phenol based reagent during initial extraction and precipitation in 8M LiCl (method 3c) was best in terms of quality and quantity as well as amplification efficiency during PCR analysis in comparison to others used protocol. Addition of PVP possibly helps to prevent oxidation of polyphenols and precipitate the phenol contaminant during extraction (Salzman et al., 1999). Moreover, β-mercaptoethanol might have inactivated ribonucleases by breaking disulfide linkages; therefore addition of 2% (w/v) β-mercaptoethanol during extraction ensures inactivation of protein (Dawson et al., 1986). LiCl solution effectively precipitates the RNA only and eventually facilitating the removal of remaining amount protein and polysaccharide contaminants (Barlow et al., 1963). Relatively faint bands developed by the RNA extracted through method 3b as compared with that of 3c was possibly associated with protein contamination due to deficiency of β-mercaptoethanol during extraction. Moreover, invisible bands produced by the RNA extracted through method 3a indicated both contamination as well as degradation of RNA. RNA extracted through method 3a was found to be worst in terms of quality and quantity as well as PCR amplification efficiency. Relatively less discrete bands of 28S rRNA and18S rRNA developed by the RNA extracted through CTAB-LiCl based method 2 revealed possible degradation of 28S rRNA and 18S rRNA (Fig. 1). Moreover, absence of discrete 28S rRNA and18S rRNA also indicated the presence of contamination in the RNA extracted through tris-saturated phenol based method 1.

Table 1: Quality and quantity of the isolated RNA

Method	A 260/280	A 260/230	Yield of total RNA (μg/g fresh weight of tissue)
1	1.89 ± 0.51	2.05 ± 0.05	66.5 ± 1.25*
2	1.81 ± 0.07	1.93± 0.10	62.33 ± 1.7
3a	1.72 ± 0.04	1.87± 0.14	53.34 ± 1.5
3b	1.91 ± 0.18	2.11 ± 0.50	69.5 ± 2.25
3c	2.01 ± 0.15	2.15 ± 0.35	75.66 ± 2.01

*Values are the mean ± standard deviation of five replicates

Table 2: Q-PCR efficiency

Methods	Minimum average C_T value of cDNA (stock)	Amplification efficiency (%)	Amplification factor
1	27.34	116.50	2.16
2	30.67	183.46	2.83
3a	32.7	231.56	3.32
3b	25.67	105.91	2.06
3c	22.85	98.27	1.98

Q-PCR analysis and amplification efficiency

This experiment was carried out to ascertain the correlation between high quality RNA and amplification effectiveness during RT-PCR analysis. Quantitative real time polymerase chain reaction studies on the expression of 26S rRNA revealed that quality of RNA was significantly connected with the 26S rRNA gene expression study. It was found that the cycle threshold value (Table 2) was lowest in the RNA (from which the cDNA has been synthesized) extracted through method 3c (indicated by black arrow in fig. 2) indicating towards the competence of the method for pure quality RNA isolation. Moreover, during investigations with method 3b and method 1 it was noticed that amplification curves (indicated by red and blue arrows respectively in fig. 2) were almost close to that of method 3c. Therefore, RNA extracted through method 3b and method 1 was also good enough to study the expression of 26S rRNA gene. However, there was an increase in the cycle threshold value was noticed during the gene expression study with the RNA extracted using method 2 (indicated by violet arrow in fig. 2), indicated the reduction of the effective amplification due to degradation of RNA. Extraction of RNA using method 3a was found to be most unsuitable for downstream application. Highest cycle threshold value (Table 2) that was noticed through the use of the RNA extracted using method 3a (indicated by green arrow in fig. 2) pointed out towards the inefficiency of this method. Furthermore, gel

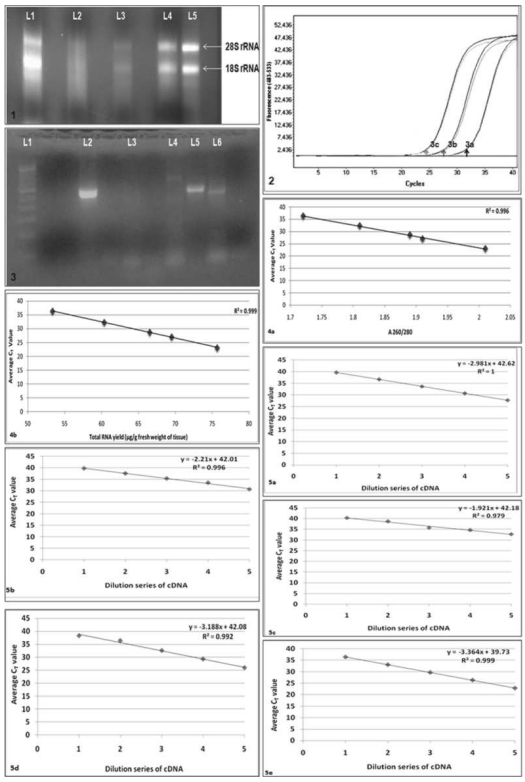

Figure 1: Gel image of extracted RNA using different method (lane 1 – method 1, lane 2 – method 3, lane 3 – method 2, lane 4 – method 3b, lane 5 – method 3c); Figure 2: Amplification curves showing different CT value that indicates change in amplification efficiency due to RNA quality; Figure 3: Agarose gel image of the amplified product of 26S rRNA indicate correlation between pure quality RNA and amplification efficiency in PCR (lane 1 – 100 bp ladder , lane 2 - method 3c, lane 3 – method 3a, lane 4 – method 2, lane 5 – method 3b, lane 6 – method 1); Figure 4a: Correlation between C_T value and RNA quality, Figure 4b: Correlation between C_T value and RNA yield; Figure 5: Determination of Q-PCR efficiency in different experimental sets, (5a – method 1, 5b – method 2, 5c – method 3a, 5d – method 3b, 5e – method 3c)

electrophoresis study of RT-PCR product of 26S rRNA gene in lane 2 (Fig. 3) also revealed the efficiency of method 3c for high quality RNA isolation. Interestingly, correlation coefficient value between RNA quality and C_T value (R^2 = 0.996) as well as RNA yield and C_T value (R^2 = 0.999) also indicate the significance of high quality RNA in Q-PCR

analysis (Figs. 4a, 4b). Data from the present research revealed that Q-PCR efficiency is highly influenced by quality of RNA (Table 2). Most efficient amplification was exhibited by method 3c, as it was noticed that the amplification efficiency (98.27%) and amplification factor (1.98) were closed to the standard limit of highly efficient PCR amplification (amplification efficiency within 90% - 100% and amplification factor = 2). Moreover, it was noticed that amplification efficiency (105.91%) and amplification factor (2.06) in method 3b was close to the standard limit of efficient PCR. However, data revealed that RNA quality was absolutely inappropriate in other three methods such as method 1, 2 and 3a for efficient PCR amplification (Figs. 5a, 5b, 5c, 5d and 5e).

Conclusion

The results of this work help us to conclude that RNA extracted using method 3c is most amenable to downstream application like Q-PCR and can be attempted for other plant species.

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